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Demonstration of Multiple Antigenic Determinants on 'Mycoplasma pneumoniae' Attachment Protein by Monoclonal Antibodies

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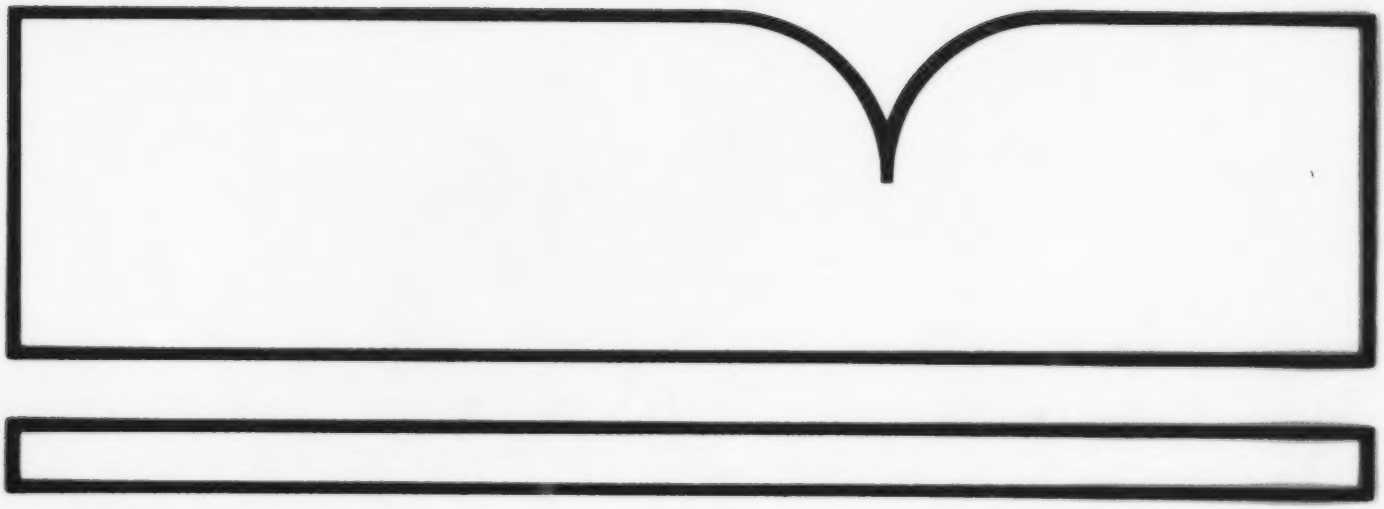

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Distinct multiple antigenic determinants of the attachment protein of Mycoplasma pneumoniae have been identified by limited proteolytic cleavage using specific monoclonal antibodies. Western blots prepared from the gels containing the cleaved fragments were probed with antiserum against M. pneumoniae or monoclonal antibodies. Five distinct bands with intact antigenic determinants were detected by the antiserum of which two bands were each reactable with two monoclonal antibodies. A sequential binding assay suggested that these monoclonal antibodies recognized different antigenic sites of each band. These results demonstrate the existence of multiple antigenic sites on attachment protein and the described procedures should prove useful for identifying those antigenic sites critical to the specific attachment of M. pneumoniae.

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Demonstration of Multiple Antigenic Determinants on *Mycoplasma pneumoniae* Attachment Protein by Monoclonal Antibodies

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Distinct multiple antigenic determinants of the attachment protein of *Mycoplasma pneumoniae* have been identified by limited proteolytic cleavage, using specific monoclonal antibodies. Western blots prepared from the gels containing the cleaved fragments were probed with antiserum against *M. pneumoniae* or monoclonal antibodies. Five distinct bands with intact antigenic determinants were detected by the antiserum, of which two bands were each reactable with two monoclonal antibodies. A sequential binding assay suggested that these monoclonal antibodies recognized different antigenic sites of each band. These results demonstrate the existence of multiple antigenic sites on the attachment protein and describe procedures that should prove useful for identifying those antigenic sites critical to the specific attachment of *M. pneumoniae*.

Previous studies on the interaction of *Mycoplasma pneumoniae* with respiratory epithelium indicated that the specific attachment of viable mycoplasmas to the target host cells was a prerequisite for the initiation of infection (9). Subsequently, it was demonstrated that a surface protein, designated P1, was involved in the attachment process (10). Using a monoclonal antibody specific to P1, we showed direct evidence that the protein was located on the surface of the attachment tip of *M. pneumoniae* (7); the same antibody also inhibited attachment. This finding has been subsequently confirmed by Feldner et al. (6) and Baseman et al. (1), using monoclonal and monospecific antibodies, respectively. Mild trypsin treatment of *M. pneumoniae* abolished its attachment capacity, cleaved P1 protein, and resulted in the appearance of P3 protein at a lower-molecular-weight region (10). However, trypsin treatment did not destroy the antigenic determinants of P1 protein recognized by the monoclonal antibody (M218) used in our later study (7). Monoclonal antibody M218 also reacted, in addition to P3, with a faint band of very low molecular weight which was thought to represent a portion cleaved from P1. This observation led to the suggestion that P1 molecules probably possess several repeating amino acid sequences which form multiple identical antigenic determinants (7). On the other hand, biological activities other than attachment have been attributed to P1 protein (5, 17). Feldner et al. (6) reported that a monoclonal antibody specific to P1 protein inhibited the gliding motility of *M. pneumoniae*, and studies of hemadsorption of *M. pneumoniae* by nonadhering mutants (5, 14) indicated that P1 was lost in some of the hemadsorption-negative mutants. It is possible that these biological activities are mediated by different sites of P1 molecules. Elucidation of the structural-functional relationships of P1 protein by epitope mapping may lead to the identification of antigenic determinants relevant to the mechanism of attachment and also contribute to a better understanding of other biological activities attributed to P1. The present report demonstrates the existence of multiple distinct determinants to P1 protein by the use of a collection of monoclonal antibodies.

MATERIALS AND METHODS

Organisms. *M. pneumoniae* M129-B7 (ATCC 29342) was used in this study. Cultures were maintained in Edward-Hayflick medium supplemented with 20% agamma horse serum, 10% yeast dialysate, and penicillin (1,000 U/ml) (4, 8). Monolayer cultures were grown in glass prescription bottles. After incubation at 37°C for 36 to 48 h, the medium was decanted, and the organisms were rinsed three times with phosphate-buffered saline (PBS), pH 7.2. The organisms were then scraped into PBS, pelleted by centrifugation (12,000 × *g* for 15 min), and processed for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis or used for immunization.

Antiserum and monoclonal antibodies. Antiserum against *M. pneumoniae* organisms was produced in rabbits by procedures described previously (16).

Hybridomas secreting monoclonal antibodies to P1 protein of *M. pneumoniae* were produced by hybridization of spleen cells from BALB/c mice immunized with *M. pneumoniae* and SP2/0-Ag14 myeloma cells (ATCC CRL-1581) by described procedures (7, 18). In another experiment, spleen cells were obtained from mice immunized with the P1 band sliced off from a 7% SDS-gel. Hybridomas that produced antibodies against P1 protein were initially screened by a solid-phase radioimmunoassay and then confirmed by protein blot in combination with a radioimmunobinding technique (12). Details of the solid-phase radioimmunoassay have been described elsewhere (13). Monoclonal antibodies in hybridoma culture fluids were concentrated by precipitation with ammonium sulfate (45% saturation) and dialyzed extensively against PBS, pH 7.4, in the cold. Concentrations were adjusted to approximately 10 mg/ml, and sodium azide was added (0.01%). The monoclonal antibodies were stored at −20°C.

Limited proteolysis of P1 protein in SDS-gels. Discontinuous SDS-gel electrophoresis was performed by Laemmli's method (15). Limited proteolytic cleavage of P1 protein separated on SDS-gels was carried out by the procedure of

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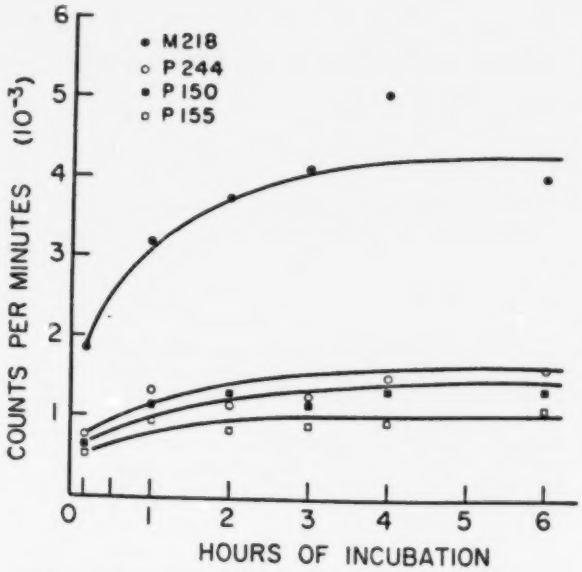

FIG. 1. Saturation curves of monoclonal antibodies. Four monoclonal antibodies specific to P1 of *M. pneumoniae* were tested for times required to saturate the solid-phase *M. pneumoniae* antigen by a radioimmunoassay (12). M218 has the highest affinity for the antigen, while all four monoclonal antibodies saturate the antigen by 4 h of incubation.

Cleveland et al. (2). P1 protein bands separated on a 7% SDS-gel stained with Coomassie blue were cut off carefully with a razor blade, trimmed to 5-mm width, and soaked in 10 ml of 0.125 M Tris hydrochloride buffer, pH 6.8, containing 0.1% SDS, 1 mM EDTA, and 10% glycerol for 30 min. The gel slices were pushed to the bottom of the individual slots of the second gel (4% stacking gel and 12% separation gel). Spaces around the slices were filled by overlaying each slot with 10 μl of the same buffer containing 20% glycerol. Finally, 10 μl of this buffer containing 10% glycerol and 2.5 μg of *Staphylococcus aureus* V-8 protease was overlaid into each slot, and electrophoresis was performed in the normal manner except that the current was turned off for 30 min when the bromophenol blue dye reached the bottom of the stacking gel. The stacking gel was 3.5 cm in height and the separation gel was 7 cm in height.

Preparation of nitrocellulose blots and immunoradioautography. Proteins separated on SDS-gels by electrophoresis were transferred to nitrocellulose filters as described previously (12). To identify bands which retained the antigenic determinants, nitrocellulose blots were incubated with rabbit immune serum or monoclonal antibodies and imaged with ^{125}I-labeled goat anti-rabbit immunoglobulin G or goat anti-mouse immunoglobulins (9). Radioautographs were produced with X-Omat-AR5 film (Eastman Kodak Co.) at −80°C, using an intensifying screen (Du Pont Co.).

Sequential binding assay. The sequential binding assay was modified from the solid-phase radioimmunoassay (13). To determine whether or not two monoclonal antibodies bind to the same epitope, the antibodies were added sequentially to the antigen in microtiter wells followed by the ^{125}I-labeled goat anti-mouse immunoglobulins. Basically, a significant rise in radioactivity counts will indicate that the two monoclonal antibodies bind to two distinct sites, whereas a sequential incubation with two monoclonal antibodies specific to the same site will result in no increase in radioactivity counts. A preliminary experiment was carried out to construct the saturation curves for the four monoclonal antibodies used in this study (Fig. 1). The results indicated that all four monoclonal antibodies saturated the antigens after about 4 h of incubation. Therefore, a 4-h incubation time was chosen for the sequential binding assay. Following the incubation of the first monoclonal antibody for 4 h, the microtiter wells were washed three times with PBS containing 2% agamma horse serum, pH 7.2; then the second monoclonal antibody was added to individual wells and incubation was continued for another 4 h. After that, the wells were rinsed two times with the PBS-agamma horse serum and then incubated with ^{125}I-labeled rabbit anti-mouse immunoglobulins (5×10^5 cpm in 50 μl) for 1 h. Finally, the wells were washed four times with PBS and swabbed with cotton applicators, and the radioactivity was determined in a gamma counter.

RESULTS AND DISCUSSION

When the P1 protein band of *M. pneumoniae* cut off from a 7% SDS-gel was subjected to limited *S. aureus* V-8 protease cleavage and re-electrophoresed on a 12% SDS-gel, in addition to a major band representing the *S. aureus* V-8 protease (Fig. 2, lane C), a number of faint bands were visualized after staining with Coomassie blue (Fig. 2, lane B); P1 remained as a single band if the V-8 protease was omitted (Fig. 2, lane A). These observations indicate that P1 protein was cleaved by the *S. aureus* V-8 protease.

To determine whether antigenicity was preserved in these protease-cleaved fragments, they were transferred to nitro-

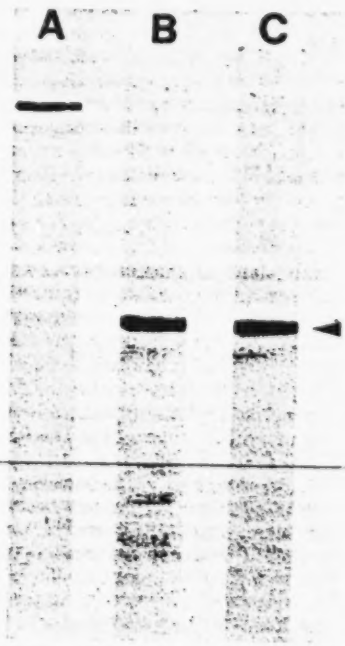

FIG. 2. Cleavage of P1 protein with *S. aureus* V-8 protease. Lane A, Untreated P1 migrated as a single band; lane B, Protease-cleaved fragments of P1 migrated to the lower region of the gel; lane C, *S. aureus* V-8 protease only, serving as a negative control. Gels were stained with Coomassie blue.

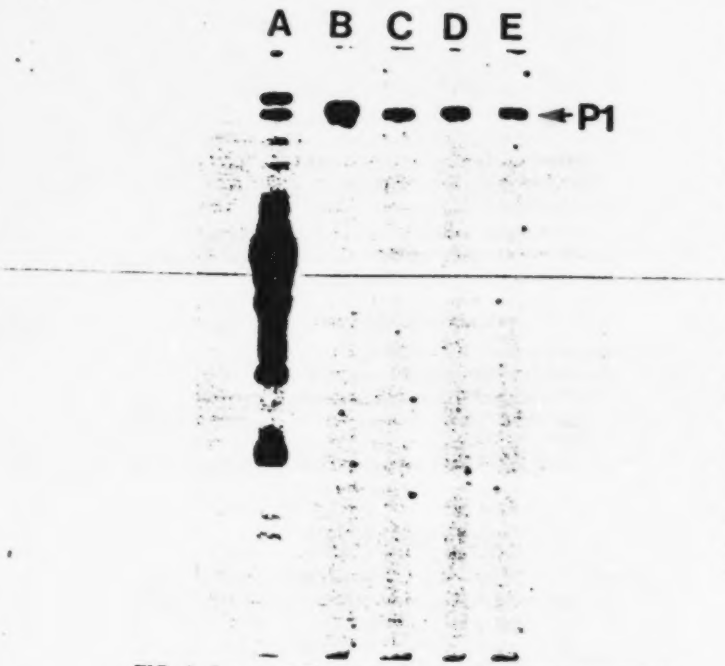

FIG. 3. Immunoradioautographs of *M. pneumoniae* proteins separated on a 10% SDS-gel and transferred to nitrocellulose filters. Lane A, Incubated with rabbit immune serum and then imaged with ^{125}I-labeled goat anti-rabbit immunoglobulin G. Lanes B to E, Same preparation as in lane A, incubated with monoclonal antibodies M218, P150, P155, and P244, respectively, and then imaged with ^{125}I-labeled rabbit anti-mouse immunoglobulins. Results indicated that all four monoclonal antibodies were specific to P1 protein.

cellulose filters and incubated with rabbit anti-*M. pneumoniae* immune serum or monoclonal antibodies. The specificity of the monoclonal antibodies is shown in Fig. 3. All four monoclonal antibodies used in this study were directed specifically against P1. Five bands were detectable with rabbit immune serum (Fig. 4). Among the five bands, band 1 reacted with monoclonal antibodies M218 and P244 and band 4 reacted with monoclonal antibodies P150 and P155. Therefore, bands 1 and 4 must possess antigenic determinants different from each other. The failure of the four monoclonal antibodies to react with bands 2, 3, and 5 indicates the absence of the corresponding antigenic determinants on bands 2, 3, and 5. However, the present data cannot rule out the possible existence of unidentified antigenic determinants which may be common to these different P1 fragments. Likewise, no conclusion can be made as to whether bands 2, 3, and 5 share a common antigenic determinant(s) or not.

Although both M218 and P244 reacted with band 1 and P150 and P155 reacted with band 4 it is still possible that either or both band 1 and band 4 possess two distinct antigenic determinants. To examine this possibility, a sequential binding assay was carried out. Sequential incubation of the solid-phase *M. pneumoniae* antigen with two monoclonal antibodies (shown to bind to two different bands in Fig. 4) resulted in significant increases in radioactivity counts compared with double incubation with a single monoclonal antibody (Table 1). This additive effect obviously is due to the binding of nonrelated monoclonal antibodies to distinct antigenic determinants on P1 molecules. However, when two monoclonal antibodies shown to react with the same protease-cleaved fragments were used, different results were observed. For example, sequential incubation of M218/P244 produced no additive effect over that of M218/M218, implying that the two monoclonal antibodies might react with the same antigenic determinant. In contrast, when the sequence of incubation was reversed, P244 followed by M218, a definite additive effect was observed, suggesting the existence of two distinct antigenic determinants on band 1. Similar results were obtained for the two monoclonal antibodies, P150 and P155, which reacted with band 4.

These results complicated our interpretation. Nevertheless, two explanations could account for the observations which favor the existence of two distinct antigenic determinants on both bands 1 and 4. The first possibility is that M218 and P244 probably bind to two different, but closely spaced, sites with such structural configuration that the site for P244 is less accessible to the antibody molecules. Consequently, the binding of M218 may interfere with the subsequent binding of P244 by steric hindrance. Primary binding by P244 did not inhibit the binding of M218. An alternate explanation is that the binding of M218 triggered a configurational change

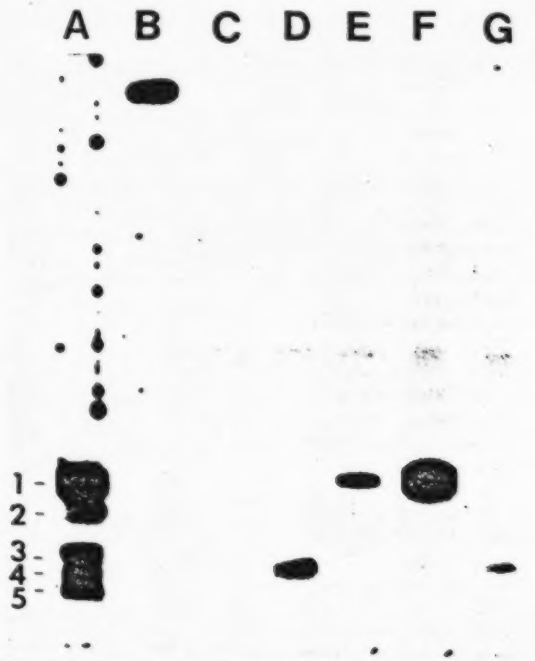

FIG. 4. Immunoradioautographs of *M. pneumoniae* P1 protein following protease treatment. P1 protein bands were cut off from a 7% SDS-gel, digested with *S. aureus* V-8 protease, re-electrophoresed on a 12% SDS-gel, and transferred to nitrocellulose filters. Lane A, Protease-digested P1 incubated with rabbit immune serum and imaged with ^{125}I-labeled goat anti-rabbit immunoglobulin G; five bands with intact antigenic determinants were revealed. Lane B, Untreated P1 migrated as a single band, indicating the purity of P1. Lane C, Blank control with *S. aureus* V-8 protease only; the faint bands represent nonspecific binding. Lanes D through G, Protease-digested P1 incubated with monoclonal antibodies M218, P150, P155, and P244, respectively, and imaged with ^{125}I-labeled rabbit anti-mouse immunoglobulins.

TABLE 1. Sequential binding assay[a]

Incubation	cpm
M218/M218	2,988
M218/P244	2,287
M218/P150	3,730
M218/P155	5,052
P244/P244	1,105
P244/M218	3,042
P244/P150	1,628
P244/P155	1,689
P150/P150	1,156
P150/M218	4,511
P150/P155	2,401
P150/P244	2,145
P155/P155	953
P155/M218	3,827
P155/P150	1,175
P155/P244	2,330

[a] Details of the experimental procedure are described in Materials and Methods. As shown in Fig. 4, both M218 and P244 recognized band 4, and P150 and P155 recognized band 1.

and as a result blocked the site for P244. These explanations may also apply to P155 and P150.

In summary, five distinct antigenic determinants of the P1 protein of *M. pneumoniae* have been identified by limited proteolytic cleavage in combination with a sequential binding assay, using specific monoclonal antibodies. This approach appears to be suitable for antigenic analysis of water-insoluble membrane-bound protein molecules which are difficult to purify. However, the combined use of SDS-gel–immunoblotting and limited protease cleavage for the identification of antigenic determinants of P1 is not without shortcomings. It is very probable that some of the antigenic determinants on P1 molecules have been destroyed by either the protease cleavage or the treatment with SDS and 2-mercaptoethenol during the sample preparation. Thus, the antigenic determinants identified by the monoclonal antibodies and rabbit antiserum in the present study were essentially those resistant to SDS and 2-mercaptoethanol. It is important to be aware that the elucidation of the entire antigenic structure of P1 will require the application of other techniques. Nevertheless, we believe many more antigenic determinants can be demonstrated on P1 molecules when additional monoclonal antibodies are produced and other proteases or chemical cleavage treatments are used.

Because of the important role that P1 protein plays in the pathogenesis of *M. pneumoniae* infection (3, 5, 9) and the recent demonstratjon that P1 is a major immunogen (11), the significance of fine analysis of the antigenic composition of P1 could be severalfold. First, it could provide information useful in the investigation of the molecular basis of the specific attachment of *M. pneumoniae* organisms to the respiratory epithelium. Second, the precise identification of an immunogenic determinant(s) relevant to this critical step of *M. pneumoniae* infection and the determination of its amino acid sequence could lead to the development of an oligopeptide vaccine. In addition, the physical basis of other biological activities, i.e., gliding motility and hemadsorption, which have been attributed to P1 protein remains unclear. It is likely that a large macromolecule such as P1 (molecular weight, 190,000) could possess a number of biological activities mediated by different "sites" of the molecule. To define the molecular basis for each of these biological activities by elucidation of the structural-functional relationships of P1 protein is an extremely difficult and challenging task. However, the utilization of monoclonal antibodies corresponding to distinct antigenic determinants to block these biological activities in experimental systems may be helpful in determining whether these biological activities attributed to P1 protein are mediated by different sites of P1 molecules. Such information should further define the role(s) of P1 in the pathogenesis of *M. pneumoniae* disease.

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